United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	The role of autophagy in mediating air pollutant induced pulmonary injury and metabolic impairment in rats
LAPR Number:	18-01-001
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 /RTP/USEPA/US
Date Originated:	12/19/2014
LAPR Expiration Date:	01/31/2018
Agenda Date:	01/14/2015
Date Approved:	01/29/2015
Date Closed:	11/29/2017

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 /RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	01/29/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6 TP/USEPA/US by Exemption 6 /RTP/USEPA/US	01/29/2015	DMR all comments addressed	

Administrative Information

1. Project Title (no abbreviations, include species):
The role of autophagy in mediating air pollutant induced pulmonary injury and metabolic impairment in rats

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information
a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
ACE111 and 158
b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/EPHD/CIB[redacted]/2015-001(Autophagy-AHC)-r1

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* [redacted] Exemption 6/RTP/USEPA/US	EPHD *Branch* CIB	MD

4. Alternate Contact:

Alternate Contact	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6 *Lotus Notes Address* [redacted] Exemption 6/RTP/USEPA/US	EPHD *Branch* CIB	MD

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the

continuation. Please spell out all acronyms and abbreviations with their initial use.

In this project we will investigate the contribution of the autophagy process in mediating air pollution-induced injury and systemic metabolic impairment using a rat model. Autophagy is a cellular process by which a starving cell under stress or injury, reallocates nutrients from unnecessary processes to more-essential processes. It is the process by which cells recycle waste material and repair themselves. It reduces the negative effects of aging and reduces the incidence and progression of aging related diseases. It is a tightly regulated process by which cell growth, development, and homeostasis are maintained by balancing the synthesis, degradation, and subsequent recycling of cellular products. Autophagy mediates the removal of reactive cytosolic waste encapsulated in a double membranous structure called the autophagosome. The unwanted cellular materials then combine with lysosomes which contain enzymes to digest and degrade these materials. In this process the digested materials can be reused by the cell for producing energy. The autophagy flux refers to the process of formation of membrane structures around the waste material, its fusion with the lysosome and subsequent degradation of cellular waste. It maintains cellular homeostasis through providing necessary nutrients to cells during stress and inducing an immune response that prepares cells in fighting for pathogens. Any cellular injury or stress is associated with activation of the autophagy process. Limited studies have been done to examine the role of this process in air pollutant-induced lung injury and systemic effects. Although increases in some autophagy-related proteins have been shown after air pollutant exposures such as ozone and particulate matter, the measurement of autophagy flux has not yet been demonstrated. The flux or the movement of autophagosomes can be measured by drugs which inhibit fusion of cellular cargo with lysosomes. We believe that autophagy is induced by air pollution exposure and is central in the subsequent tissue repair process. Furthermore enhancing or reducing this process by pharmacological treatment, can alter pollutant-induced injury and repair processes. The overarching plan for this project is to examine if autophagy plays a role in air pollution-induced systemic metabolic and inflammatory health effects, and determine if manipulating autophagy response might offer a potential therapeutic option for those exposed to high levels of pollutants.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

To examine the contribution of air pollution in systemic health effects and the contribution of autophagy response in multiple organs such as liver, muscle and lung, animal experiments are necessary. The interactive effects of pollutants that encounter the lung on multiple organ systems require studies using whole organisms and systems approach. We have used different strains of rats to determine cardiovascular and extrapulmonary effects of air pollutants. The use of rat models is necessary in order to examine complex mechanisms associated with cardiopulmonary and metabolic interactions. In vitro culture studies will not allow one to integrate the process of autophagy and systemic metabolic impairment.

b. Justify the species requested:

National Institute of Health guidelines recommends the use of rats to study human cardiovascular and metabolic diseases. Rat has been also a preferred animal model for the study of air pollution health effects. Moreover metabolic disorders are better modeled in rats than in mice or in lower vertebrates. There are adequate genetic and protein data base for rats and reagents available to study systemic effects. Toxicology data are available for rats to correlate findings of air pollution health effects. We have done a number of toxicological studies using Wistar Kyoto (WKY) rats and have characterized injury and metabolic aspects. Since proposed studies involve continuation of our previous studies using WKY rats for examining systemic health effects, we propose using these rats to understand how metabolic effects and injury/inflammation are modulated by the process of autophagy in response to air pollution exposure.

3. How was it determined that this study is not unnecessary duplication?

Literature searches on 1/20/15 in Pubmed for all cited literature from 1970s until today, and the peer reviewed toxicology and clinical literature provide the evidence that proposed studies are not duplicative. Specifically, only a few studies have been done using air pollutants to show changes in autophagy markers but the determination of sequential changes in autophagy process- autophagy flux is not studied. No air pollution studies have been done in Wistar based models to understand interaction of autophagy, metabolism and pollution induced injury and inflammation in multiple organs. We have the current knowledge, through direct interaction with scientists in the field and attending scientific meetings, and believe that our experiments are not duplicated anywhere else.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

In this project we will examine autophagy specific markers in tissues following air or ozone exposure in rats pretreated with pharmaceutical agents known to induce or suppress autophagy response, and correlate autophagy to inflammation, and metabolic effects. Male Wistar Kyoto (WKY) rats (12 week old) will receive two sequential intraperitoneal injections of appropriate vehicles or drugs as stated below. One hour after the second injection of selected drugs, rats will be exposed to air or 1.0 ppm ozone for 4 hours and the interactive effects of the drugs and ozone will be assessed at 0, 4 and 24 hours after the termination of exposure. The ozone concentration is selected based on our other metabolic studies where a robust metabolic response in terms of glucose intolerance was observed without an overt ozone induced lung injury and inflammation. We selected this concentration of ozone to relate findings of autophagy response to metabolic impairment. Rats will be necropsied at these time points to determine lung injury and inflammation, systemic metabolic effects and changes in tissue markers of autophagy for lung, liver and muscle. Tissue samples will be collected, and later assessed for circulating free fatty acids and lipids, and metabolic and stress hormone levels such as insulin, leptin and cortisol.

This study will be divided in two experiments, one for determining the effect of ozone when autophagy is inhibited by chloroquine and second for determining the effect of ozone when autophagy is induced by leupeptin (the experimental protocol schematic indicating timing and treatments/exposures is attached). The concentration of chloroquine and leupeptin are selected based on previous rat studies where autophagy responses have been successfully manipulated by using these concentrations of drugs (Liu et al., 2015; Long et al., 2013; Sun et al., 2013; Bolli et al., 1983; Freeman and Lloyd, 1983; Saito et al., 1992) (all papers attached).

Experiment 1: Male WKY rats will be intraperitoneally injected with pharmaceutical grade saline or pharmaceutical grade chloroquine prepared in saline (60 mg/ml) at 1ml/kg 15 hour and 1 hour prior to the start of air or 1.0 ppm ozone exposure. After 4 hours of exposure rats will be euthanized and necropsied at 0, 4 and 24 hour after the termination of exposure (n=6/group x 2 treatments x 2 exposure conditions x 3 time points=72 rats in total).

Experiment 2: Male WKY rats will be intraperitoneally injected with 0.5 % dimethyl sulfoxide (DMSO) in pharmaceutical grade saline (vehicle) or leupeptin prepared in 0.5 % dimethyl sulfoxide (DMSO) vehicle (36 mg/ml) at 1ml/kg, 15 hour and 1 hour prior to the start of air or 1.0 ppm ozone exposure. After 4 hours of exposure rats will be euthanized and necropsied at 0, 4 and 24 hour after the termination of exposure as indicated in experiment 1 (n=6/group x 2 treatments x 2 exposure conditions x 3 time points=72 rats in total).

In addition to these two experiments pertaining to air pollution exposure and autophagy modulation, we will include a positive control assessment of autophagy in tissues. This will be important for us in determining the relative induction of autophagy as it relates to a known autophagy inducer-mediated activation of autophagy response. Rapamycin has been shown to induce autophagy in animals and has been extensively used to induce autophagy response. We will intraperitoneally inject 6 additional rats with 4 mg/kg rapamycin dissolved in 1 mL of 0.5% pharmaceutical DMSO and 6 rats with 0.5% DMSO vehicle as indicated in experiment 2 (injected 1mL/kg body weight). Rapamycin will be injected twice at zero hour and at 15 hours, and 1 hour later chloroquine (60 mg/mL in saline given 1 mL/kg body weight) will be intraperitoneally injected in these 12 rats to accumulate autophagosomes in tissues. Thirty minutes after chloroquine injection, rats will be necropsied and lung liver and muscle tissues will be isolated and preserved for determination of autophagy markers as a positive control. (12 rats total)

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

We propose to use 6 rats per group for all exposures that is minimally required for statistical evaluation. This number of rats will be required for standard analysis of variance techniques. We like to emphasize that a highly conservative experimental design is adapted which will allow us to look at many different biomarkers in

the same animal without using multiple sets of animals. We also will be sharing tissues with other investigators (brain and plasma) in order to reduce the use of animals. Another example of effective use of animals is that we collect blood for all clinical markers from same animals for which one lung is used for molecular assessment and the other lung for lavage. The lung, liver and muscle as well as aorta and heart tissues from same animal will be used for determining autophagy flux and biomarkers of injury. Liver, muscle and adipose tissues are additionally used for metabolic markers. We will also bank various tissue samples for potential future use. The tissues collected from positive control groups will be used for all comparative experiments related to autophagy.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	156	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:		

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

A. General whole body inhalation exposure conditions

During air or 1.0 ppm ozone inhalation exposures of maximum of 4 hours/day (whole-body), rats will be placed in individual stainless steel wire mesh cages, and food and water are withheld while the rats are being exposed. The rats will be weighed prior to and following exposure during exposure days and examined for any visible clinical signs of discomfort or poor health. The rats will also be checked after each exposure when they are returned to home cages. All findings are recorded. Ozone exposures will be done using whole body exposure system.

B. Intraperitoneal injection of saline as vehicle: Rats will be injected with 1 ml/kg pharmaceutical grade saline twice, intraperitoneally at 15 and 1 hour prior to the start of exposure.

C. Intraperitoneal injection of Chloroquine in saline: Rats will be injected with 1 ml/kg chloroquine suspension in pharmaceutical grade saline (60 mg/ml) twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. This dose of chloroquine is used in many studies related to autophagy and other experiments. The pH of chloroquine saline suspension will be determined. The literature using single multiple chloroquine injections daily in rats at or near the dose level proposed in our study (some papers attached) have not indicated peritonitis complications. Our study will involve a maximum of 2 day time to necropsy. Regardless, we will inspect the peritoneal cavity for potential inflammatory reaction or damage and if any indication of peritonitis is observed, we will consult attending veterinarian for alternative injection strategy.

D. Intraperitoneal injection of 0.5% DMSO in pharmaceutical grade saline as vehicle: Rats will be injected with 1 ml/kg 0.5% DMSO in saline twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. Since the final concentration of DMSO injection will be 0.5%, we think that viscosity might not be an issue, regardless, if there is a problem with viscosity when using 25g needles for injection, we will consider using 23 g needles with luer lock syringes.

E. Intraperitoneal injection of leupeptin in 0.5% DMSO saline suspension: Rats will be injected with 1 ml/kg leupeptin (36 mg/ml) prepared using 0.5% DMSO in pharmaceutical grade saline twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. This dose of leupeptin is used for studies involving autophagy in rats and mice.

F. Intraperitoneal injection of rapamycin 4 mg/kg/ml DMSO (0.5%): Rats will be injected intraperitoneally twice

with rapamycin (at zero and 15 hours). This dose of rapamycin is used in many studies involving induction of autophagy (please see attached papers).

b. Survival Blood Collections (method, volume, frequency):
none
c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
Respiratory monitoring using whole body Plethysmography - EMKA Technologies system: All rats from each experiment will be monitored for breathing parameters using EMKA system (equivalent to Buxco system used in many previous studies under approved LAPRs). Breathing parameters are monitored in freely moving rats. No restraint is used. Rats are placed in plethysmography chambers while pressure parameters are collected to compute breathing frequency, minute volume, respiratory time and enhanced pause before and after exposures. The rats are placed in a whole-body plethysmography for 5-10 min. No restraint or other stresses are involved in this process. This testing will be done prior to the first exposure, and 3 hour or 23 hour after exposure prior to necropsy for all 144 animals. This measurement allows in depth evaluation of lung health in unrestrained freely moving rats.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:
none
e. Breeding for experimental purposes (e.g. length of pairing, number of generations):
none
f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:
The drug treatment will occur for a maximum of two times. After the first treatment rats will be monitored for 2-3 hours for possible discomfort. Then during the second treatment in the morning prior to exposure rats will be monitored for possible discomfort and signs of weight loss. During exposure Exemption 6 Exemption 6Exemption 6Exemption 6 will monitor animals, at least once per hour for entire exposure duration. During post exposure period of up to 20 hours, rats will be monitored in the evening and then in the morning for visible signs of discomfort and weight loss by Exemption 6Exemption 6 Exemption 6 All animals will be monitored for signs of illness (huddling, isolation with ruffled exterior, shivering, development of hindered movement, etc) and if any adverse effect is observed, we will consult with the staff veterinarian and follow the recommended protocol. Visual inspection of labored breathing and isolation will be carefully monitored. No weekend treatment or exposures are scheduled, and the animals will be necropsied within 48 hours of the start of the drug treatment (please see attached protocol), so animals will not be monitored during weekend for the potential drug effects. No weight loss or significant distress is expected in any of the experimental conditions.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).
a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):
none
b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):
none
c. Testing methods:
n/a
d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):
n/a
e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

n/a

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

n/a

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

n/a

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

none

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

n/a

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

n/a

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

n/a

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

n/a

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

We do not expect any overt toxicity that result in clinical signs of lethargy, distress, pain or weight loss. Nevertheless, the attending Veterinarian will be consulted and recommendations will be followed for animals displaying unexpected weight loss of 10% or greater or showing any distress during experiment. Animals will be weighed daily during the experimental period of 48 hours.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Animals will be weighed daily during experimental period. If weight loss of >10% occurs overnight, animals will be isolated in a clean control atmosphere and observed for recovery trend, and may be reused for the study if recovered. All animals will be monitored each day during and after exposure and any animals displaying signs of illness (huddling, isolation with ruffled exterior, shivering, development of hindered movement, etc) will be considered for euthanasia as per advice of the staff veterinarian. Visual inspection of labored breathing and isolation will be carefully monitored. No deleterious effects of these non-surgical procedures are expected.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

n/a

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 156

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 156

2. Species (limited to one per LAPR): Rat(s)

3. Strain: WKY rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

none

4. Sources of animals:

Charles River Laboratories Inc.

5. Provide room numbers where various procedures will be performed on animals:

1. Rats will be housed in one of the animal housing room upon arrival (Exemption 6 Exemption 6 Exemption 6- or other available room) and during non exposure periods.

2. During exposure rats will be transferred in an original rack with rats housed in home cages to green floor inhalation exposure rooms (Whole body plethysmography - room Exemption 6 Exemption 6 Exemption 6; whole body exposures - Exemption 6 Exemption 6 Exemption 6). Once the exposure and measurements are complete rats will be transferred to their home cages in the same rack and moved back to animal holding room.

3. The day of necropsy animals will be transferred to Exemption 6 Exemption 6 for necropsy using transfer cages with beta chips bedding and filtered cage tops.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

no ***Room Numbers:*** n/a

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

none

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

none

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

none

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage

(e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

All animals will be housed 2/cage with beta chips bedding in A building during non exposure periods. Enviro-dri® nesting material will be used in cages for enrichment.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1. Ozone: Ozone exposure will occur in whole body exposure chamber to a maximum of 1.0 ppm concentration. The LC50 for ozone is 4.8 ppm in rats. Health and safety protocol for ozone is available and will be followed during exposure (HSRP #778; Title: Small Animal Inhalation Exposures to Nitrogen Dioxide; includes ozone protocol). No deleterious effects of ozone are expected. All animals will be monitored continuously during exposure as indicated in B.8.b.

2. Intraperitoneal injection of saline as vehicle: Rats will be injected with 1 ml/kg pharmaceutical grade saline twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. No health and safety protocol is required for saline. No deleterious effects of saline are expected.

3. Intraperitoneal injection of pharmaceutical solid chloroquine diphosphate in pharmaceutical grade saline: Rats will be injected with 1 ml/kg chloroquine suspension in pharmaceutical grade saline (60 mg/ml) twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. No health and safety protocol is required for chloroquine. No deleterious effects of chloroquine are expected. Chloroquine diphosphate - Oral LD50 (rat): 623 mg/kg and intraperitoneal LD50 value is 102 mg/kg.

4. Intraperitoneal injection of 0.5% pharmaceutical DMSO in pharmaceutical grade saline as vehicle: Rats will be injected with 1 ml/kg 0.5% DMSO in saline twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. LD50 value of DMSO in rats is 9.9 mL/kg intraperitoneal. The contact with DMSO is expected to be none or incidental, nevertheless, double nitrile gloves will be used and changed if any contact occurs.

5. Intraperitoneal injection of leupeptin (Calbiochem) in 0.5% DMSO saline suspension: Rats will be injected with 1 ml/kg leupeptin (36 mg/ml) prepared using 0.5% pharmaceutical DMSO in pharmaceutical grade saline twice, intraperitoneally at 15 and 1 hour prior to the start of exposure. Pharmaceutical grade leupeptin is not available. Many other studies have successfully used the Calbiochem chemical in this testing. Leupeptin oral (rat) LD50 720 mg/kg; subcutaneous (rat) LD50 1100 mg/kg; intravenous (rat) LD50 80 mg/kg.

6. Intraperitoneal injection of rapamycin 4 mg/kg/ml DMSO (0.5%): Rats will be injected intraperitoneally twice with rapamycin (at zero and 15 hours). Rapamycin (ApexBio Inc), an antibiotic is available in powder form which will be dissolved in DMSO and diluted with saline to result in 4mg/mL 0.5% DMSO. Pharmaceutical grade rapamycin is not available as an injectable preparation in desired concentration of DMSO. Rapamycin at or above 0.1% is not identified as possible human carcinogen. No LD50 data are available. Doses at 5 mg per kg or higher are used for daily administration in rodents without any toxicity associated with the use of rapamycin.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

1. Saline: Pharmaceutical grade

2. Chloroquine: Pharmaceutical grade

3, DMSO, 0.5% in saline: Pharmaceutical grade.

4. Leupeptin (Calbiochem) in 0.5% pharmaceutical DMSO saline suspension: No pharmaceutical grade Leupeptin is available. Many other studies have successfully used the Calbiochem chemical in this testing.

5. Rapamycin (Apex Bio Inc) in 0.5% pharmaceutical DMSO saline suspension: Pharmaceutical grade rapamycin is not available as an injectable preparation in desired concentration of DMSO.

6. Ozone: Ozone is generated for inhalation exposures for toxicity testing and no pharmaceutical grade is available for inhalation exposure.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

no

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

n/a

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols and oversee the experiment. Assist in study planning, animal handling, testing and necropsy	Twenty years of experience working with rats at EPA and other institutions, all required training completed.
Exemption 6 Exemption 6 Exemption 6 Exemption 6	Technical Staff	Prepare protocols and oversee the experiment. Assist in study planning, animal handling, testing and necropsy	Twenty years of experience working with rats at EPA and other institutions, all required training completed.
Exemption 6	Student	Assist in study planning,	Has nearly two years of experience handling

		animal handling, testing and necropsy	animals. Has completed required training. Exemption 6 Exemption 6 has taken hands-on rat training. Exemption 6 will train and continue to mentor Exemption 6 Exemption 6 for animal handling procedures.
Exemption 6	Student	Assist in study planning, animal handling, testing and necropsy	Has three years of experience handling animals. Has completed required training. Exemption 6 Exemption 6 has taken hands-on rat training. Exemption 6 will continue to mentor Exemption 6 Exemption 6 for animal handling procedures.
Exemption 6	Post-Doc	Assist in study planning, animal handling, testing and necropsy	Has three years of experience handling animals. Has completed required training.
Exemption 6	Technical Staff	Assist in study planning, animal handling, pulmonary physiological testing	Twenty years of experience working with rats at EPA and other institutions, all required training completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year n/a

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Animals will necropsied for blood sample and tissue collections following terminal anesthesia, at 1hour, 4 hour or 24 hour after the exposure.

2. Describe the euthanasia techniques:

Method(s): Anesthesia plus exsanguination

Agent(s): Pentobarbital injectable preparations, diluted with sterile saline to achieve maximum of 200mg/ml concentration

Dose (mg/kg): Maximum 150-250 mg pentobarbital/kg

Volume: maximum 3.0 ml

Route: intraperitoneal

Source(s) of information used to select the above agents/methods:

- Veterinary Staff
- IACUC

3. Provide justification and references for any euthanasia agent or method that is not consistent with

recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).
n/a

4. Describe how death is to be confirmed.
Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Transferred to another study
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	12/19/2014

Submitted: 12/19/2014

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	01/08/2015	Exemption 6 *Lotus Notes*	EPHD *Branch*	MD *Submitted to Branch*

	by Exemption 6 Exemption 6/RTP/USEPA/US	***Address*** Exemption 6 Exemption 6/RTP/USEPA/US	CIB	***Chief for Approval*** 12/19/2014 06:52 PM

ATTACHMENTS

18-01-001 PI resp.pdf 18-01-001 DR rev.pdf Autophagy 2015 Andres experiment schematic.pptx

Zhang et al 2013, rapamycin 4 mg per kg in rat.pdf Wang et al 2013 rapamycin 5 mg per kg in rat.pdf

Houssaini et al 2013 rapamycin 5 mg per kg rat.pdf Saito et al, 1992, leupetin 33 mg per kg in rats.pdf

Sun et al, rat and chloroquine and multiple ip 50 mg per kg.pdf Long et al, Choloquine 50 mg per kg in rats.pdf

Liu et al, Cholorquine 60 mg peer kg in rats.pdf Freeman and Lloyd, 1983, leupeptin 50 mg per kg in rats.pdf

Bolli et al, 1983, leupeptin 40 mg per kg in rats.pdf

Actions

First Update notification sent: 11/30/2015
Second Update notification sent: 01/19/2016
First 2nd Annual notification sent: 12/09/2016
Second 2nd Annual notification sent: 01/04/2017
1st Expiration notification sent:
2nd Expiration notification sent:

History Log: